SECOND TRIANNUAL REPORT (YEAR 3)

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EVALUATION OF DRIED STORAGE OF PLATELETS FOR TRANSFUSION: PHYSIOLOGIC INTEGRITY AND HEMOSTATIC FUNCTIONALITY.

Principal Investigator: Arthur P. Bode, Ph.D.
East Carolina University
School of Medicine

Attachments: Report from subcontract principal investigator, Marjorie S. Read, Ph.D., The University of North Carolina at Chapel Hill.

Abstract accepted for poster at the annual meeting of the American Society for Hematology, Dec 2 - 6, 1994

Administrative Activity:

The collaboration with Dr. Blajchman of McMaster University on testing lyophilized platelets in hemostatically compromised rabbits is proceeding with vigor (see results summary below). An abstract containing data from these studies and analysis of similar preparations in the Xylum Clot Signature Analyzer has been accepted for poster presentation at the annual meeting of the American Society of Hematology, Dec. 2-6, 1994, at Nashville, TN.

One of the previously mentioned manuscripts has now been accepted as a paper for publication in the Proceedings of the National Academy of Sciences (PNAS). The other is in final preparation of figures for submission to the journal Blood. At least two more manuscripts are planned. The abstract for the American Association of Blood Banks attached to the previous triannual report has been accepted for oral presentation, Nov. 12-17, 1994, at San Diego, CA. In the week of Sept. 12-16, a review of all progress since the inception of our work on lyophilized platelets (1988 - present) was presented at the British Blood Transfusion Society in Southampton, England.

Scientific Progress:

The current long term dried storage study was terminated at 8 months in this reporting period. The preparations under study were (1) 0.02% permanganate in 5% BSA, and (2) 1.8% paraformaldehyde in 500 mM Trehalose stored desiccated at RT, 4°C, or at -70°C. Neither prep maintained good morphology at any temperature, and there was minimal ristocetin aggregation or hypotonic shock response remaining. At this 8 month workup, yield of intact platelets upon reconstitution was dependent on the storage conditions: 27-29% for RT, 41-46% for 4°C, 68-78% for -70°C. Our conclusion from this study reinforces what we learned from earlier storage studies: that there is significant deterioration of product at RT. In this particular experiment we found that permanganate-treated platelets, or para-platelets dried in Trehalose are as susceptible to loss of integrity over time as other preps. Our platelet handling techniques have improved since these studies were initiated. Another set of new preparations will be set aside for long-term storage in the next reporting period.

This is the first period in which we can report results of the bleeding time test after infusions of human reconstituted platelets into thrombocytopenic rabbits under the agreement with Dr. Blajchman. A total of 12 dried platelet preparations processed as PARA 21 or PARA22 or PARA45 (1.8% paraformaldehyde for 45 min.) were sent for his analysis after preliminary experiments to reestablish the model. Some of the same preparations were tested in the Xylum Clot Signature Analyzer, also. The results are summarized in the attached abstract. The data show that the lyophilized platelets had a lower % recovery in the rabbits after infusion, but corrected the prolonged bleeding time like fresh platelets in a manner consistent with the platelet count obtained. More tests are underway to establish statistically any differences in effectiveness that may exist among the groups.

The CSA data on these preparations for Dr. Blajchman showed an in vitro bleeding time and collagen-induced thrombus formation equivalent to that of fresh

platelets. We have now studied a total of 19 lyophilized platelet preparations in the CSA after recombining with washed RBC and citrated PPP recalcified with 5 mM Ca^{2+}. In general summary, it appears that the PARA21 and PARA45 preparations give the most normal results, while PARA22 platelets (1.8% paraformaldehyde, 2 hours) performed somewhat less well but better than 6-8 day old expired platelet concentrates from the blood bank. The few permanganate (0.02%) preparations tested to date (n = 3) gave a highly variable response inbetween fresh platelets and expired PC. We conclude from these findings that each of these lyophilized preparation techniques can produce hemostatically competent platelets, although process variables may influence function in ways not yet discerned.

We have continued to test the adhesion of reconstituted lyophilized platelets in the Baumgartner annular perfusion chamber. In this reporting period, we have run 20 Baumgartner experiments on lyophilized platelets or controls under various conditions, including modulation of platelet microparticle count in the plasma or addition of inhibitor substances such as glycocalicin to investigate mechanisms involved in adhesion phenomenae. We have learned that filtration of platelet microparticles from the plasma used in these experiments reduces adhesion of the fresh, lyophilized, or blood bank platelets. Also, it appears that PGE-1 does not reduce platelet adhesion, and that aprotinin actually enhances adhesion. The purpose of these experiments is to ascertain the dominant mechanisms of platelet adhesion to the vessel in this system and then assure that the lyophilized platelets work in the same way.

For this reporting period, we have processed 29 units of human blood for preparation of lyophilized platelets. Most were dried in large volume bottles and sent to Dr. Blajchman for efficacy testing in the thrombocytopenic rabbit model, or to Chapel Hill for testing in their rat bleeding time models. More data will be available from these studies in the next reporting period. Please find attached the subcontract progress report from Dr. Read and colleagues.

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CITY Greenville STATE/PROVINCE: North Carolina
COUNTRY USA ZIP/POSTAL CODE: 27858
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HEMOSTATIC PROPERTIES OF HUMAN LYOPHILIZED PLATELETS IN A THROMBOCYTOPENIC RABBIT MODEL AND A SIMULATED BLEEDING TIME DEVICE.

A. P. Bode, M. Blajchman, L. Bardossy*, and M.S. Read. Departments of Pathology, East Carolina University, Greenville, NC, and McMaster University, Hamilton, Ontario, and University of North Carolina at Chapel Hill, NC.

We have shown previously that our preparations of lyophilized human platelets (Lyo-Plt) are [illegible]ally intact upon reconstitution, and that they adhere to thrombogenic [illegible] in vitro and in vivo (Blood 82:159a, 1993). Now we have tested the ability of these preparations to correct the ear bleeding time (BT) in rabbits made thrombocytopenic and immunosuppressed as detailed elsewhere (Blood 82:3489, 1993). Also, we have measured the in vitro bleeding time (IVBT) and collagen-induced thrombus formation (CITF) of Lyo-Plt in a prototype in vitro bleeding time device called the Clot Signature Analyzer (CSA, Xylum Corp, NY). In the rabbits, the endogenous platelet count was $\leq 10 \times 10^6$/mL and the BT was ≥ 900 seconds before infusion of 40-50 x 10^6 platelets. A platelet count and duplicate BTs were then performed one hour after infusion. For the CSA test, platelets were resuspended in fresh citrated plasma at 300-500 x 10^6/mL and combined with an equal volume of washed RBC to remake whole blood. $CaCl_2$ was added to 5 mM in the blood just prior to initiating each run. Mean results for Lyo-Plt versus human platelet-rich plasma (fresh) or 5-8 day old expired blood bank platelet concentrates (Exp PC) are tabulated below.

	Rabbit Model				Clot Signature Analyzer		
	n	% Recovery	Count	Ear BT	n	IVBT	CITF
Lyo-Plt	(12)	58%	108	234 sec.	(4)	118 sec.	73%
Fresh	(6)	79%	153	177 sec.	(4)	134 sec.	88%
Exp PC	(0)	ND	ND	ND	(3)	281 sec.	39%

The Lyo-Plt had reduced 1 hour recovery in the rabbit model relative to fresh platelets, but the mean BT result was similar to the value seen in non-infused rabbit controls with equivalent endogenous platelet counts. In the CSA, the Lyo-Plt gave results similar to that of fresh platelets and better than that of stored platelets in the IVBT and the CITF (t-test, $p < 0.05$). These findings support the notion that Lyo-Plts are hemostatically active.

Accepted for poster

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Report period: June-September 1994
University of North Carolina at Chapel Hill

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Performance Site: University of North Carolina at Chapel Hill
Principal Investigator: Marjorie S. Read, Ph.D
Co-PI: Robert Reddick, MD

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Progress report for June-September 1994

The hemostatic potential of circulating rehydrated platelets (Specific aim #1)

We have continued our work on the development of a radioactive labeling method for rehydrated and fresh platelets for use in hemostatic and thrombogenic studies. We have been successful in radiolabeling rehydrated platelets with the Zynaxis ^{125}I-PKH95 compound, with over 90% of the label remaining in association with platelets after 24 hours of incubation in normal dog plasma. Labeling with this agent is necessary since we have shown that commonly used radiolabels in fresh platelets are lost from rehydrated platelets (see previous progress reports). However, the commercial production of ^{125}I-PKH 95 has recently been discontinued. We have obtained permission from the manufacturer to produce the compound in our own laboratory, and are currently preparing to do so. Once we have generated the compound, we will characterize it and continue with our *in vivo* studies using radiolabeled rehydrated and fresh platelets.

We have used the drug cyclophosphamide in normal dogs to induce thrombocytopenia. Although high doses of this drug induce thrombocytopenia readily in dogs, the side effects of the treatment are severe and may be life threatening to the animal. There are also other pathological changes that occur with treatment with this drug that have not yet been fully characterized. In order to develop a more suitable animal model for hemostatic studies, we have started investigating a rat model. We have been successful in inducing thrombocytopenia in rats by treatment with an anti-rat thrombocyte antibody. With antibody treatment in a normal rat, we typically see a drop in platelet count from 600,000-1,000,000/μl to less than 25,000/μl with a concomitant lengthening of toenail bleeding time from 2-3 minutes to greater than 15 minutes. This model is advantageous for several reasons; we can control the platelet concentration in the rat by modulating the amount of antibody used for treatment, cheap, easy to ge into the vasculation, model has been used for a TTP model (Sanders, 1988) In several experiments, the infusion of rehydrated platelets into the thrombocytopenic animals causes an increase in the platelet count and a return of the bleeding time to normal. We are continuing refinement of this model and will be using it in the future for other hemostatic studies with rehydrated platelets.

As relayed in the previous report, we have acquired the Stago Diagnostica ST4 clotting machine. We have used this machine to compare the clotting times of fresh and rehydrated platelets in the form of PRP (or R-PRP for rehydrated PRP). Clotting times of PRP and R-PRP are typically 80.4 +/-1/4 seconds and 87.9+/-2.0 seconds (see previous report). We are currently using this machine to examine changes in clot formation with the addition or subtraction of rehydrated platelets and with combinations of fresh and rehydrated platelets. Other clotting experiments starting in the lab include the measurement of the tensile strength of clots formed in the presence of rehydrated platelets, and the amount of clot retraction that clots formed in the presence of rehydrated platelets undergo.

We have used scanning electron microscopy (SEM) to examine clots formed in the presence of rehydrated platelets. SEM micrographs of these clots show rehydrated platelets in association with fibrin strands throughout the clot with an appearance and periodicity of fresh platelets in a normal clot. We are continuing these studies and will be investigating the effects of the fibrinolytic system on clots formed in the presence of rehdyrated platelets (Specific aim #2).